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Full paper

Overexpression of Na^+/Ca^{2+} exchanger 1 display enhanced relaxation in the gastric fundus

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ABSTRACT

In gastric smooth muscles, the released Ca^{2+} activates the contractile proteins and Ca^{2+} taken up from the cytosol cause relaxation. The Na^+/Ca^{2+} exchanger (NCX) is an antiporter membrane protein that controls Ca^{2+} influx and efflux across the membrane. However, the possible relation of NCX in gastric fundus motility is largely unknown. Here, we investigated electric field stimulation (EFS)-induced relaxations in the circular muscles of the gastric fundus in smooth muscle-specific NCX1 transgenic mice (Tg). EFS caused a bi-phasic response, transient and sustained relaxation. The sustained relaxation prolonged for an extended period after the end of the stimulus. EFS-induced transient relaxation and sustained relaxation were greater in Tg than in wild-type mice (WT). Disruption of nitric oxide component by N-nitro-L-arginine, EFS-induced transient and sustained relaxations caused still marked in Tg compared to WT. Inhibition of PACAP by antagonist, EFS-induced sustained relaxation in Tg was not seen, similar to WT. Nevertheless, transient relaxation remained more pronounced in Tg than in WT. Next, we examined responses to NO and PACAP in smooth muscles. The magnitudes of NOR-1, which generates NO, and PACAP-induced relaxations were greater in Tg than in WT. In this study, we demonstrate that NCX1 regulates gastric fundus motility.

1. Introduction

In the gastrointestinal tissues, relaxations of smooth muscles are caused by Ca^{2+} taken up from the cytosol. This process of relaxation requires Ca^{2+} clearance, resulting that the free intracellular Ca^{2+} concentration must decline (1–3). This Ca^{2+} clearance requires the transport of Ca^{2+} out of the cytosol by pathways involving the plasma membrane Ca^{2+}-ATPase, the Na^+/Ca^{2+} exchanger (NCX) and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (4,5). Among gastrointestinal tissues, relaxation rather than contraction has big power in the gastric fundus. In the feature of the gastric fundus, electrical field stimulation (EFS) induces more powerful sustained relaxation than any other intestinal regions (6–8). This sustained relaxation stored ingested food, relating to a physiological role of stomach (9).

NCX is an antiporter membrane protein that controls Ca^{2+} influx and efflux across the membrane. The functional roles by which NCX influences gastrointestinal motility including contraction and relaxation are incompletely understood and vary by tissue, although its role in cardiac muscle and brain neurons is well understood. Our group is showing to reveal the functional role of NCX on gastrointestinal motility because Ca^{2+} homeostasis is central to the regulation of gastrointestinal smooth muscle functions (10–12). In previous article, we examined the response to EFS in the longitudinal smooth muscles of the distal colon in smooth muscle-specific NCX1.3 transgenic mice (Tg). We found that the amplitudes of EFS-induced relaxation that persisted during EFS were

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greater in Tg than in wild-type mice (WT) (13). In next previous article, we investigated the response to EFS in the longitudinal smooth muscles of the ileum in Tg. We found that the amplitudes of EFS-induced phasic and tonic contractions were greater in Tg than in WT (14). Similar to EFS, the magnitude of ACh-induced contraction was greater in Tg than in WT (14). However, the involvement of NCX1 overexpression in the relaxation of the gastric fundus has not been clarified. In the gastric fundus, we found that both EFS-induced transient relaxation and sustained relaxation were larger in NCX1 heterozygote knockout mice (HET) and NCX2 HET than in WT (12). In this previous article, we showed that NCX1 and NCX2 expressed in myenteric neurons, but not smooth muscles, affect the relaxation and control motility in the gastric fundus (12).

These findings prompted us to investigate whether NCX1 overexpression affects the gastric fundus motility. To better understand the stored function of gastric fundus, we characterized in using circular muscle strips isolated from the gastric fundus. Moreover, we tried to recognize the component underlying altered motility.

2. Materials and methods

2.1. Drugs

Atropine and N-nitro-L-arginine (L-NNA) were purchased from Wako Pure Chemical (Osaka, Japan). NOR-1 [(±)-(E)-Methyl-2-[(E)-hydroxyamino]-5-nitro-6-methoxy-3-hexaneamide] was purchased from Dojin (Kumamoto, Japan). PACAP and PACAP6-38 were purchased from the Peptide Institute (Osaka, Japan).

2.2. Smooth muscle-specific NCX1.3 transgenic mice

Tg were produced as reported previously (15). These mice on the C57BL/6 background were used to compare to WT in all analyses. All procedures used in this study were performed according to the institutional policies of the Osaka Prefecture University Animal Care and Use Committee.

2.3. Organ-tissue bath system

We recorded EFS-induced responses using previously described methods (16) with some modifications (12). Briefly, the fundus was taken from mice (9–12 weeks old). We pulled the fundus strips in the direction of the circular muscle layer. We used isotonic force transducers (TD-112A; Nihonkohden, Tokyo, Japan) to detect EFS-induced responses. Clearly, the fundus strips were stimulated with EFS (10 Hz) for 30 s. The fundus strips were treated with atropine (1 μM), L-NNA (30 μM) and PACAP6-38 (1 μM) in advance of the EFS. Transient and sustained relaxations were analyzed by measuring the extent of the maximal relaxations in response to Ca^{2+}-free EGTA solution.

2.4. RNA isolation and real-time PCR

Total RNA was extracted from fundus with previously described methods (17,18). The primers used for amplification of NCX1 were as follows: 5′-CCTTGTGCATCTTAGCAATG-3′ and 5′-TCTCACTCATCTCCACCAGA-3′. The primers used for amplification of PAC1 mRNA variant 1 and PAC1 mRNA variant 2 were as follows: 5′-CTGCGTGCAGAAATGCTACTG-3′ and 5′- AGCCGTAGAGTAATGGTGGATAG-3′; 5′- TACTGTGTGTGTAACTGTGTGGG-3′ and 5′- GCCAGCCGTAAGTAGATGCTC-3′. The results were normalized to endogenous HRPT expression levels.

2.5. Statistical analysis

The results are expressed as the means ± S.E. Statistical significance was determined using one-way ANOVA for non-repeated measures to detect differences among WT and Tg in the absence and presence of drugs. The differences between groups were determined using the Tukey–Kramer test. The statistical significance of the parametric data was evaluated using a two-tailed Student's *t*-test to detect differences between WT and Tg. A P value of less than 0.05 was considered significant.

3. Results

3.1. Overexpression of NCX1

We initially investigated the overexpression of NCX1 in the fundus of Tg using quantitative real-time PCR. As shown in Fig. 1, NCX1 mRNA was overexpressed in the fundus of Tg at a two-fold higher level compared to endogenous NCX1.

3.2. EFS-induced responses

We investigated the response to EFS in the fundus of WT and Tg. Fig. 2A provides typical recording charts of responses to EFS. EFS in WT caused a bi-phasic response, transient relaxation during EFS and sustained relaxation. The sustained relaxation prolonged for an extended period after the end of the stimulus. We show vertically dotted lines to separate transient and sustained relaxations. To analysis the amplitude of the transient and sustained relaxations, we used the amplitude of the maximal relaxations in response to Ca^{2+}-free EGTA solution. The magnitudes of the maximal relaxations were similar between WT and Tg (data not shown). EFS-induced transient relaxations were greater in Tg than in WT (Fig. 2B upper). Similarly, sustained relaxations were greater in Tg than in WT (Fig. 2B lower).

In response to EFS, acetylcholine as an excitatory transmitter and nitric oxide (NO) and PACAP as inhibitory transmitters are allowed to produce from myenteric neurons in the fundus. In addition, EFS caused a small contraction that was seen at exactly the same time of the EFS (Fig. 2A). We next investigated the response to EFS in the presence of atropine to recognize the mechanisms underlying enhanced relaxation in Tg (Fig. 2C). As illustrated in Fig. 2D, EFS in the presence of atropine elicited an increased transient relaxation in both WT and Tg. However, atropine has no significant effects on EFS-induced sustained relaxations

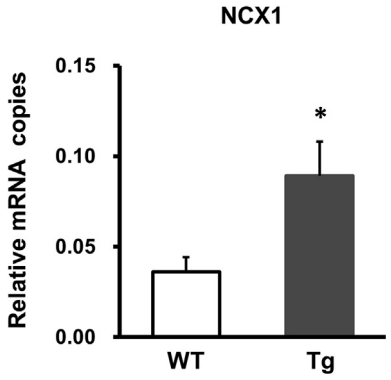


Fig. 1. NCX1 expression in the fundus. The expression level of NCX1 in WT (n = 10) and Tg (n = 13) were examined by quantitative real-time PCR. The mRNA level of NCX1 is shown as a fold increase relative to the level of HPRT mRNA. *$P < 0.05$ compared with WT using *t*-test.

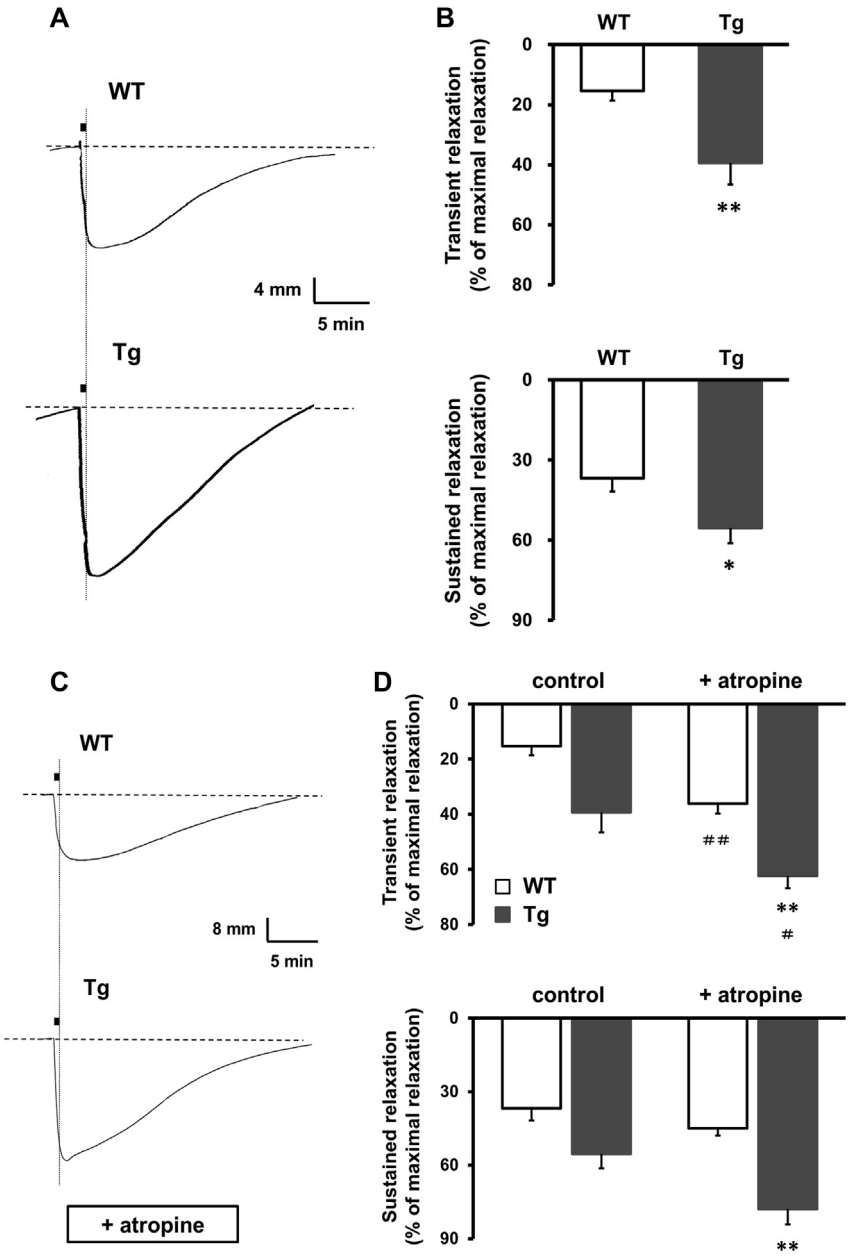
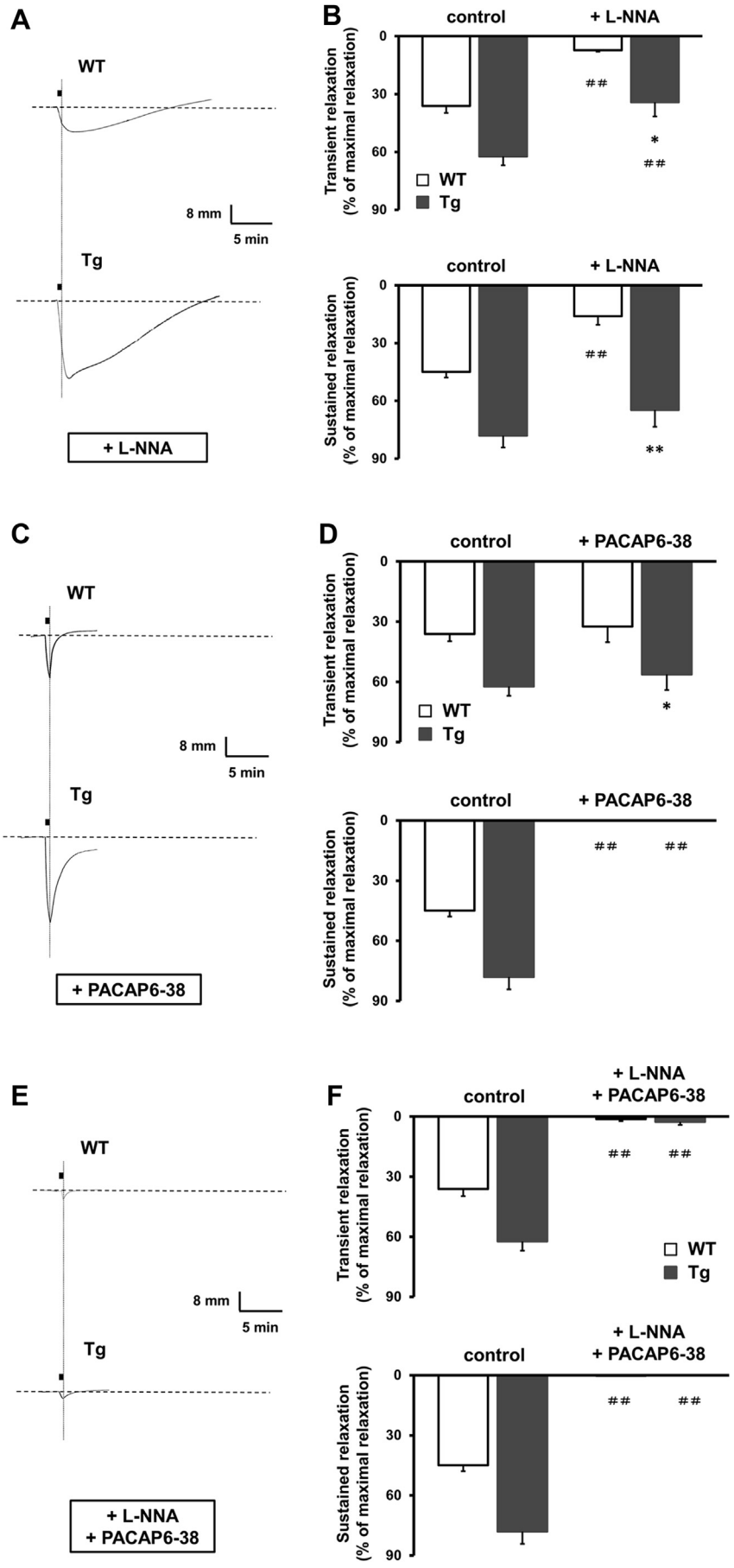


Fig. 2. Enhanced relaxations during EFS. (A, B) EFS-induced responses in circular muscle strips isolated from the fundus in WT (n = 9) and Tg (n = 4). (C, D) EFS-induced relaxation in the presence of atropine in circular muscle strips isolated from the fundus of WT (n = 7) and Tg (n = 6). (A, C) Representative recording traces of EFS-induced responses are shown. Bars indicate the duration (30 s) of EFS. Vertically dotted lines indicate a border between transient and sustained relaxations. (B, D) Quantitative data on the transient relaxations during EFS (upper) and sustained relaxations after EFS (lower). Relaxations are expressed as percentages of Ca^{2+}-free EGTA-induced relaxation. (B) $^{*}P < 0.05$, $^{**}P < 0.01$ compared with WT using *t*-test. (D) Control show data in the absence of atropine. $^{**}P < 0.01$ compared with WT and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with control using Tukey–Kramer.

in both WT and Tg. It is likely that acetylcholine can mediate the response during EFS. Even in the presence of atropine, the transient and sustained relaxations were greater in Tg than in WT. These results direct that Tg are subject to an increased relaxation.

Our previous study found that NO and PACAP act as important mediators of transient and sustained relaxation in the mouse gastric fundus (6). Indeed, we evaluated the effect of NCX1 overexpression on the EFS-induced relaxation in the presence of L-NNA, an inhibitor of NO synthase, and PACAP antagonist. The fundus strips were incubated with L-NNA to characterize the neurotransmission process, followed by EFS (Fig. 3A). The amplitudes of the transient relaxation were almost suppressed in WT and significantly suppressed in Tg (Fig. 3B upper). Nevertheless, transient relaxations remained more pronounced in Tg than in WT (Fig. 3B upper). EFS pre-treated with L-NNA significantly decreased sustained relaxation in WT but not Tg. The magnitudes of sustained relaxations were greater in Tg than in WT (Fig. 3B lower). The fundus strips were incubated with PACAP antagonist PACAP6-38 followed by EFS, EFS-induced transient relaxation in WT and Tg were similar to those observed in absence of PACAP6-38. The amplitudes of the transient relaxation were greater in Tg than in WT (Fig. 3C and D upper). Furthermore, sustained relaxations were all inhibited in WT and Tg (Fig. 3C and D lower). These results indicate that Tg directed enhanced response to NO and PACAP. Moreover, we


A
WT
8 mm
5 min
Tg
+ L-NNA
B
control
+ L-NNA
Transient relaxation (% of maximal relaxation)
0
30
60
90
WT
Tg
##
*
##
Sustained relaxation (% of maximal relaxation)
##
**
C
WT
8 mm
5 min
Tg
+ PACAP6-38
D
control
+ PACAP6-38
Transient relaxation (% of maximal relaxation)
*
Sustained relaxation (% of maximal relaxation)
##
##
E
WT
8 mm
5 min
Tg
+ L-NNA
+ PACAP6-38
F
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+ L-NNA
+ PACAP6-38
Transient relaxation (% of maximal relaxation)
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Sustained relaxation (% of maximal relaxation)
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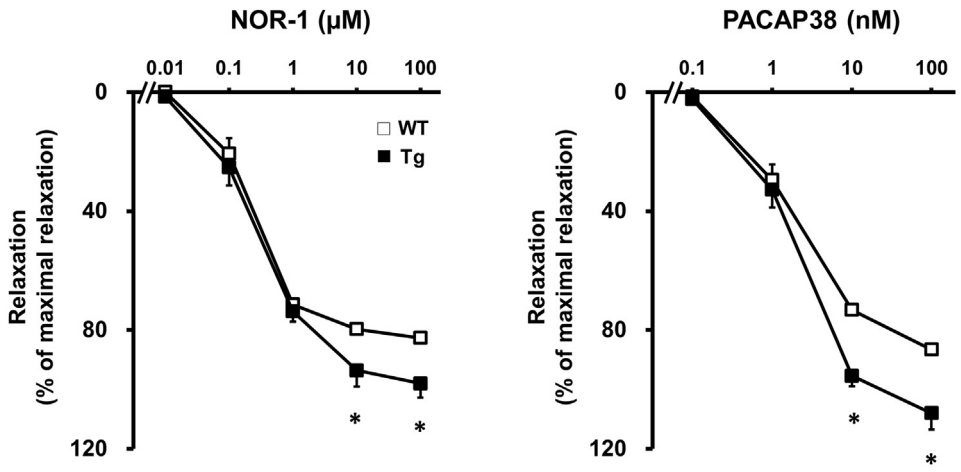

Fig. 4. NOR-1- and PACAP-induced relaxations. NOR-1-induced relaxation in circular muscle strips isolated from the fundus of WT (n = 4) and Tg (n = 4). PACAP-induced relaxation in circular muscle strips isolated from the fundus of WT (n = 4) and Tg (n = 4). NOR-1 and PACAP-induced relaxations were expressed as percentages of Ca^{2+}-free EGTA-induced relaxation. $^{*}P < 0.05$ compared with WT using *t*-test.

studied relaxations after both applications of L-NNA and PACAP6-38 (Fig. 3E). EFS pre-treated with L-NNA and PACAP6-38 completely vanished relaxations in WT and Tg (Fig. 3F).

3.3. Responses of smooth muscles

We determined the effect of NCX1 overexpression on the relaxation in response to NO and PACAP in smooth muscles. We examined the responses to five different concentrations of NOR-1, which generates NO, and four different concentrations of PACAP. NOR-1 and PACAP induced relaxation in the concentration-related manner. Moreover, the amplitudes of relaxation induced by 10 and 100 μM NOR-1 were greater in Tg than in WT (Fig. 4 left). Likewise, PACAP at 10 and 100 nM induced greater magnitudes of relaxation in Tg than in WT (Fig. 4 right). To investigate whether the expression of PAC1, a PACAP receptor, is altered in Tg, we examined and compared the expression of PAC1 using quantitative real-time PCR. The $2^{-\Delta\Delta Ct}$ values for PAC1 mRNA variant 1 were 1.0 ± 0.15 (n = 4) for WT and 0.61 ± 0.11 (n = 4) for Tg, which was not significantly different. The $2^{-\Delta\Delta Ct}$ values for PAC1 mRNA variant 2 were 1.0 ± 0.46 (n = 4) for WT and 0.67 ± 0.11 (n = 4) for Tg, which was not significantly different. Indeed, the expression of PAC1 in Tg was similar to that in WT. Therefore, NCX1 overexpression in smooth muscles can enhance the relaxations to NO and PACAP.

4. Discussion

The results in this manuscript show an interesting and important ability of NCX1 during EFS-stimulated relaxations in the gastric fundus. Specifically, we revealed that NCX1 overexpression enhanced relaxation in the gastric fundus. Clearly, our results show that NCX1 controls the gastrointestinal motility by regulating the relaxation response in the gastric fundus.

Enhanced relaxation is likely due to an increased response to inhibitory transmitter such as NO and PACAP in smooth muscles. How does NCX1 overexpression show increased response, followed by enhanced relaxation? It suggests that accelerated Ca^{2+} extrusion by NCX1 overexpression may be related to enhanced relaxation. One possible mechanism is NCX1 activation through cGMP/PKG signaling previously reported by Furukawa (19). Moreover, these Tg demonstrated increased Ca^{2+} extrusion and decreased cytoplasmic Ca^{2+} concentration in the aorta (20). We considered another possible mechanism by which Tg showed enhanced relaxation. In urinary bladder smooth muscles, these Tg showed enhanced contractions and a prolonged duration of Ca^{2+} sparks (21). In this previous findings, Tg has been hypothesized to experience increased Ca^{2+} influx via NCX, which extends the period of high Ca^{2+} levels and maintains the contraction. If NCX1 overexpression just accelerates Ca^{2+} influx, it may be not very reasonable in this study. The possibility that NCX1 overexpression enhances to extrude Ca^{2+} in the smooth muscles of the gastric fundus listens reasonable. We put emphasis on the observation that under the resting cellular ionic condition and membrane potential, NCX1 overexpression in Tg may act to extrude Ca^{2+} from the cytoplasm.

Consistent with previous findings (6), the transient relaxation in the presence of L-NNA and the sustained relaxation in the presence of PACAP6-38 were almost suppressed in WT. These results support the possibility that NO and PACAP act as important mediators of relaxations in the mouse gastric fundus, and that L-NNA or PACAP6-38 resistant relaxations were mainly mediated by PACAP or NO, respectively. In contrast, transient relaxations in the presence of L-NNA remained in Tg, although the sustained relaxation in the presence of PACAP6-38 were almost suppressed in Tg. However, EFS-induced relaxations in the presences of L-NNA and PACAP6-38 completely vanished in Tg. These data are strange and interesting results. There is a possibility that overexpression of NCX1 in the smooth muscles caused the increase in functional coupling and/or collaborative functions between NO signaling and PACAP signaling. This increase in functional coupling and/or collaborative functions between NO signaling and PACAP signaling may affect the intracellular Ca^{2+} concentration changes. As a result, EFS in the presence of L-NNA may induce transient relaxations in Tg.

Fig. 3. Components of enhanced relaxations in Tg. (A, B) EFS-induced relaxation in the presence of atropine and L-NNA in circular muscle strips isolated from the fundus of WT (n = 4) and Tg (n = 4). (C, D) EFS-induced relaxation in the presence of atropine and PACAP6-38 in circular muscle strips isolated from the fundus of WT (n = 4) and Tg (n = 4). (E, F) EFS-induced relaxation in the presence of atropine, L-NNA and PACAP6-38 in circular muscle strips isolated from the fundus of WT (n = 3) and Tg (n = 3). (A, C, E) Representative recording traces of EFS-induced responses are shown. Bars indicate the duration (30 s) of EFS. Vertically dotted lines indicate a border between transient and sustained relaxations. (B, D, F) Quantitative data on the transient relaxation during EFS (upper) and sustained relaxation after EFS (lower). Relaxations are expressed as percentages of Ca^{2+}-free EGTA-induced relaxation. Control show data in the presence of atropine. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with WT and $^{\#\#}P < 0.01$ compared with control using Tukey–Kramer.

Another unique finding emerged from our study is that EFS pretreated with L-NNA enhanced transient relaxation in Tg. Transient relaxations in the presence of L-NNA remained more pronounced in Tg than in WT, although the transient relaxation in the presence of L-NNA were almost suppressed in WT. It suggests that the response to PACAP is more increased in Tg compared to the response to NO. The following findings further support this indication. The sustained relaxation in the presence of PACAP6-38 were almost suppressed both WT and Tg.

We demonstrated that Tg show enhanced relaxation in the gastric fundus. NCX1 HET has previously displayed enhanced relaxation in the gastric fundus (12). Tg and NCX1 HET indicate same phenotypes in the viewpoint of enhanced relaxation except their mechanisms. However, NOR-1-induced relaxations were similar in NCX1 HET and WT (12). Like NOR-1, PACAP induced similar magnitudes of relaxation in NCX1 HET and WT (12). Unlike NCX1 overexpression in smooth muscles, NCX1 heterozygous deficiency in smooth muscles does not enhance the responses to NO and PACAP. In our previous study, we concluded that NCX1 HET may direct increased releases of NO and PACAP from myenteric neurons (12). In conclusion, the relaxations induced by NO and PACAP were enhanced by NCX1 overexpression. Hence, we can propose the possibility that NCX1 overexpression enhances to extrude Ca^{2+} in the smooth muscles in the gastric fundus. Since abnormal productions of NO and PACAP are caused in functional gastrointestinal disorders, the new compounds affecting on NCX could be valuable (22–24). Indeed, the functions of NCX in gastrointestinal disorders are an important theme of future study.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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